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Optimization of Biofilm Mediated Antibiotic Resistant *Lactobacillus Acidophilus* Isolated from Tooth Decay of Rural School Children's Around Tirupur District

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Abstract: Dental caries is one of the main oral problems suffered by the human population. The major constituent of biofilm other than bacterial cells is the Extracellular Polymeric Slime matrix (EPS) which is secreted by the bacterial cells themselves. The cariogenic infection caused by *Lactobacillus acidophilus,* most common pathogen in human life, causes serious problem among caries patients. Therefore 50 plaque samples were collected from tooth decay of rural school children's using forceps in and around Tirupur district. The isolates were identified using standard biochemical test. The isolates were screened using microtiter plate assay. Different conditions for better biofilm formation, antibiotic susceptibility of the selected organism was investigated and also the ability of the test pathogen to produce antimicrobial substance. Among the selected isolates, LAVG02, LAVG06, LAVG17, LAVG36, LAVG38, LAVG75 and LAVG85, LAVG92 were found as better biofilm producers and were selected for the detailed study. The strains were cultivated at 30ºC, 37 ºC and 45 ºC at the initial *p*H of 6.5 for 24 h, with the addition of different concentration (1-10%) of different carbon and nitrogen sources used resulted in different extent of increase in the bacterial growth and biofilm production. In this study glucose and yeast extract induced biofilm formation. It was found that the selected isolate was sensitive to Rifampicin; intermediate to Amoxicillin and resistant to Azithromycin, Vancomycin, Sulphamethizole, Roxithromycin, Cloxacillin, ciprofloxacin and Trimethoprim. Results of this study suggest reducing excessive consumption of sugary foods and drinks will help to prevent the occurrence of dental caries.

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Keywords: Dental caries, Biofilm, Culture conditions, Optimization, *Lactobacillus acidophilus*, antibiotic resistance.

Introduction

Dental caries is one of the most common chronic and multifactorial diseases affecting majority of adult and 60-90% of school children. The appearance of a caries lesion is determined by the coexistence of three main factors: acidogenic and acidophilic microorganisms, carbohydrates derived from the diet, and host factors.

Dental plaque is a complex biofilm formed on teeth surface composed of self-produced Extracellular Polymeric Substances (EPS), mainly glucans [1]. The fermentation of dietary sugars by acidogenic oral micro biota plays key role in the development of caries. Nowadays, lactic acid bacteria produce various compounds such as organic acids diacetyl, hydrogen peroxide and bacteriocin or bacteria proteins during lactic fermentations [2].

Infectious diseases are the most significant threat for human beings with high morbidity and mortality throughout the world, due to the rapid emergence of multiple drug resistance (MDR) it creates a major health problems in the medication of infectious diseases spread by pathogenic microbes [3].

The main factor contributing to microbial resistance is the biofilm formation by the microbes that allow them to withstand extreme environmental conditions and antimicrobial agents. The biofilm forming bacteria are resistant to antimicrobial agents due to the lack of penetration of antimicrobial agents. In this study, biofilm producing microorganisms were isolated from dental plaque. Among these a bacterial isolate *Lactobacillus acidophilus* was selected for further study investigating the different factors in biofilm formation and antibiotic sensitivity of it was assayed against different commercially available antibiotics.

Materials and Methods

Sampling

Dental plaque was collected from tooth decay of rural school children in and around Tirupur District, Tamilnadu, who were suffering from different stages of dental caries. Samples were collected with a sterile cotton swab saturated with sterile physiological saline. Then the cotton swab was poured into a screw-cap tube containing 10 ml sterilized physiological saline. The pour plate technique was applied for isolation of bacteria using nutrient agar (NA) medium.

Identification of Lactobacillus acidophilus causing oral tooth decay

The isolates were identified as *L. acidophilus* on the basis of cultural, morphological and standard biochemical tests according to Bergy's Manual of Systematic Bacteriology [4].

Production of antimicrobial compound by Lactobacillus acidophilus

Determination of Lactic Acid Production of Lactobacillus acidophilus

The strains were grown in Man Rogosa Sharpe (MRS) broth for 48 h and supernatant was collected by centrifuging at 10,000 rpm for 15min at 4°C. Phenolphthalein was added in to the 20 ml of supernatant as an indicator for titrimetric estimation. One ml of 0.1M NaOH is equivalent to 90.08 mg of lactic acid.

Determination of Hydrogen Peroxide Production of Lactobacillus acidophilus

Twenty five ml of dilute sulphuric acid were added to 25ml of MRS broth culture of test organisms. Titration was carried out with 0.1 N of Potassium permanganate. Each ml of Potassium permanganate is equivalent to 1.070 mg of H_2O_2. A decolourization of the sample was regarded as end point.

Quantification of Organic Acid Production of Lactobacillus acidophilus and Determination of its pH value

1% (v/v) of 24 h active culture of *Lactobacillus* was used to inoculate 10% sterilized skimmed milk and initial *p*H (6.6) was determined by digital electrode *p*H meter. The inoculated skimmed milk was incubated at 37°C for 72 h and sample were collected in every 24 h, 48 h, 72 h and liquid of coagulated milk were separated by filtration. The *p*H of the separated liquid was recorded using a digital electrode *p*H meter. The quantification of organic acid was performed through titration with 0.1 N NaOH using phenolphthalein as *p*H indicator.

Screening of cariogenic dental biofilm produced by Lactobacillus acidophilus

Optimization of different conditions for biofilm formation

In the present study, different factors (Incubation time, temperatures and *p*H) and the effect of different carbon and nitrogen sources in biofilm formation were studied.

Effect of carbon and nitrogen sources by Micro Titer Plate Assay

An overnight culture of bacterial strain was prepared with chemically defined media. After vortexing the overnight culture, 50µl of volume were transferred in to Micro titer plate. The basal medium MRS broth without glucose was substituted with different sugar (glucose, lactose, fructose, maltose, galactose, raffinose and sucrose) at different concentration (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%). 200µl of these media were transferred to each micro plate. Another medium was prepared with the nitrogen sources substituted with peptone, meat extract, yeast extract at different concentration. After 24 h of incubation at different temperature (25°C, 30°C, 37°C and 45°C) with CO_2 and without CO_2 medium was removed from well. The micro titer plate wells were washed two times with sterile distilled water to remove loosely associated bacteria. Each well was stained with 200µl of 95% ethanol. The ethanol was removed after 15 mins. After that plates were air dried for 45 mins. Each well was stained with 150µl of 1% crystal

violet solution for 5 mins. After staining plates were washed with sterile distilled water. At this end point biofilm were visible as purple rings formed on the side of each well. The wells were left to dry and after drying each well was stained with acetic acid 33% and the absorbance was read in an ELISA reader.

In vitro evaluation of sensitivity assay of the selected isolate against some commercially available antimicrobial agents

Determination of antibiotic sensitivity pattern

Antibiotic susceptibility was semi-quantitatively determined by disc diffusion method. The different groups of antibiotics tested were: Class I Group – Inhibitors of cell wall synthesis (Amoxicillin, Cloxacillin, Vancomycin), Class II Group – Inhibitors of protein synthesis (Azithromycin, Clarithromycin, Roxithromycin), Class III Group - Inhibitors of nucleic acid synthesis (Ciprofloxacin, Rifampicin), Class IV Group – Inhibitors of folate metabolism (Sulphamethizole, Trimethoprim). The culture was inoculated into the MRS broth which was incubated at 37°C for 12hours. Plates were made with Mueller Hinton agar and allowed to solidified 10 to 15 minutes. The 0.1ml of this culture was inoculated in the plates using L-Rod by spread plate technique. The antibiotic disks of Amoxicillin (30mcg), Cloxacillin (5mcg), Vancomycin (30mcg), Azithromycin (30mcg), Clarithromycin (15mcg), Roxithromycin (30mcg), Ciprofloxacin (30mcg), Rifampicin (30mcg), Sulfamethizole (300mcg), and Trimethoprim (30mcg) were placed in the plates. Agar plates with antibiotic disks were then incubated for 37°C for 24 hours. The diameters of the inhibition zone were measured using a ruler under a colony counter apparatus. The results were expressed as sensitive (S), marginally susceptible (I), and resistant (R).

Results

Preface

Early childhood caries is a public health problem that continues to affect babies and pre-school children worldwide. A comprehensive review of the epidemiology of ECC showed that its prevalence varies from population to population. This disease exerts a social, physical, mental and financial burden on global scale, with developing countries being the most affected.

Isolation and characterization of biofilm producing Lactobacillus acidophilus

Totally hundred caries plaque samples were collected from different dental clinic hospitals from rural area of Tirupur city, Tamilnadu, India (Fig. 1). 50 isolates of *Lactobacillus acidophilus* were isolated from the samples. Selective media: Man Rogosa Sharpe media were used to isolate the cariogenic pathogen *Lactobacillus acidophilus* (Fig.2). The decay causing predominant cariogenic pathogen *Lactobacillus acidophilus* strains were identified by comparing the results with standard biochemical tests of *Lactobacillus acidophilus*.

Fig. 1: Tooth Decay Sample

Fig. 2: Isolated colonies of *Lactobacillus acidophilus* from tooth decay

(Man Rogosa Sharpe media)

Production of antimicrobial compound by oral Lactobacillus acidophilus

Lactic acid estimation

Lactic acid production of EPS producing cariogenic plasmid strains were studied by titrimetric estimation which include MTVG08, MTVG13, MTVG14, MTVG15, MTVG22, MTVG47 and MTVG48 produce equal amount of lactic acid 180.16 mg/ml in the oral cavity of rural caries patients (Fig.3, 4).

Fig. 3: Lactic acid production of *Lactobacillus acidophilus*

C: Control; P: Positive

Fig.4: Confirmation of Lactic acid production-Titrimetric assay

Quantification of organic acid and determination of pH value

The present experiment indicates that organic acid production was increased with the incubation time. On the other hand, *p*H of the media decreased with the increasing acid production. In this study highest acidity (1.8%) was observed after 72 h of incubation at 37°C for *Lactobacillus acidophilus* isolated from caries of children (Fig. 5).

Fig.5: Organic acid production by *Lactobacillus acidophilus*

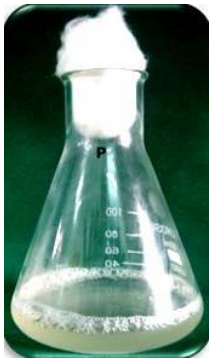
C: Control; P: Positive

Determination of H_20_2 production by oral Lactobacillus acidophilus

The finding from the present studies of H_2O_2 production by oral *Lactobacillus acidophilus*. A substantial proportion of the isolates LAVG02, LAVG20, LAVG25, LAVG38 and LAVG46 produced H_20_2, as revealed by decolourization of pink color that appeared during titration. The

highest proportion was found among all cariogenic isolates, 5 out of 5 strain (100%) being positive. Among five strains only one strain namely LAVGO2 (340.20 mg / ml) produced maximum amount of H_20_2 as compared to the other cariogenic isolates namely (LAVG20 - 255.15 mg / ml), (LAVG25 - 212.625 mg / ml), (LAVG46 - 212.625 mg / ml), and (LAVG38 – 127.575 mg / ml) (Fig. 6).

Fig.6: Hydrogen peroxide production by *Lactobacillus acidophilus*



Screening of carcinogenic dental biofilm

Optimization of different conditions for biofilm formation

In this study, different factors, different carbon and nitrogen sources in biofilm formation were studied.

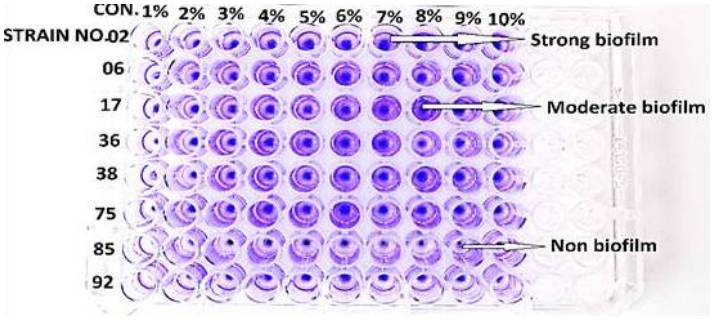
Effect of different carbon and nitrogen source on biofilm production by Lactobacillus acidophilus

In these experiments, micro titer plates were prepared similarly as in the modified method of micro titer plate assay described in screening procedure. The ability of biofilm formation assay in presence and absence of some dietary carbohydrates showed that more biofilm formation is related to presence of the sugars. The influence of temperature, pH and different concentrations of sugars on the bacterial growth and biofilm production by cariogenic isolates (LAVG02, LAVG06, LAVG17, LAVG36, LAVG38, LAVG75, LAVG85 and LAVG92) were investigated. The results indicate that the higher glucose concentrations may be positive for biofilm formation, independently of the hydrodynamic conditions in microtiter plates. Comparing the other carbon sources the enhanced biofilm formation was observed with increasing 10% concentrations of glucose in the medium but biofilm formation was then gradually diminished in the lower concentrations. This indicates that biofilm formation was increased to a certain level of glucose concentration in the medium at 37°C and the gradual diminished may be an effect of glucose concentration on the microbe at 45°C. During the investigation of response of biofilm formation of the selected isolate with the utilization of different organic nitrogenous compounds as nitrogen source, it was found that yeast extract was suitable nitrogen source for biofilm formation (Conc.

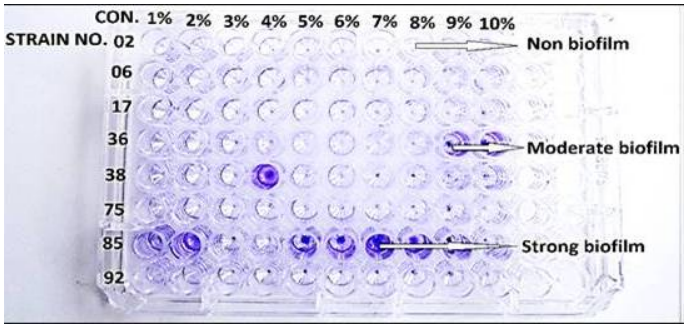
1-10%) at 37°C and biofilm formation was gradually diminished to a certain level of yeast concentration (1-10%) in the medium at 45°C (Fig.7, 8).

Fig.7: Effect of carbon source on biofilm production by *Lactobacillus acidophilus*

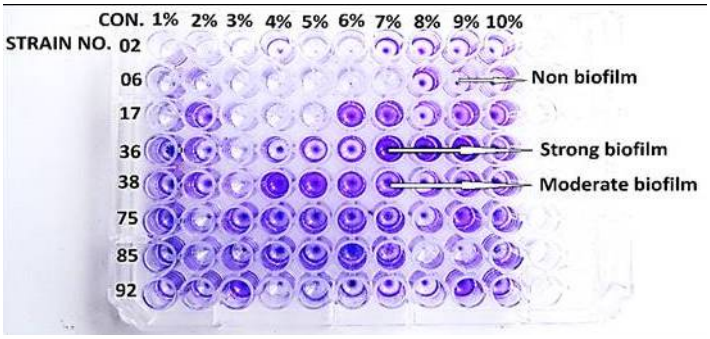
a. Effect of different concentration of Galactose on biofilm production at 30°C



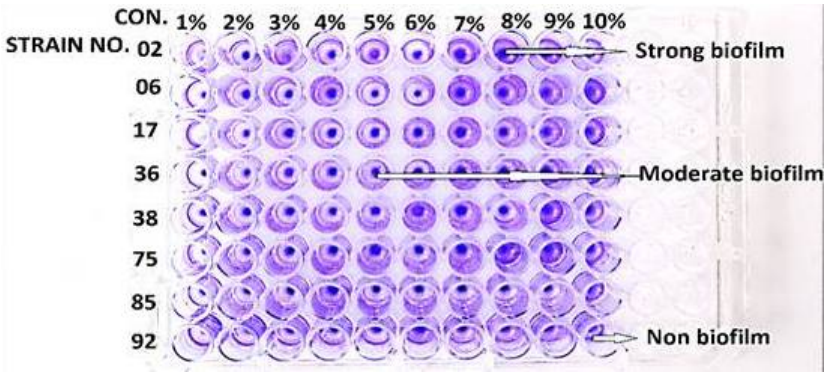
b. Effect of different concentration of Fructose on biofilm production at 30°C



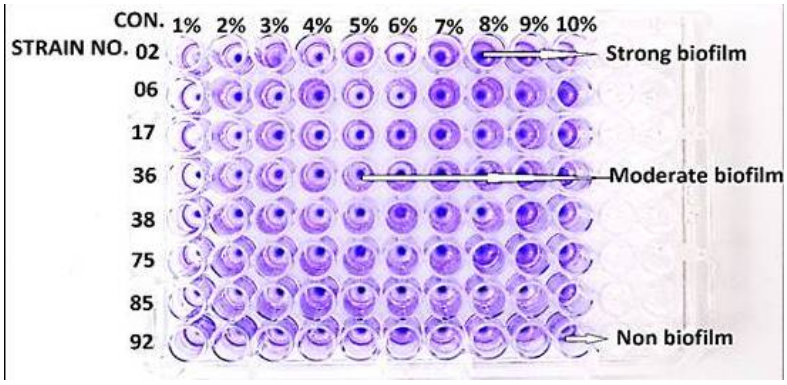
c. Effect of different concentration of Glucose on biofilm production at 30°C



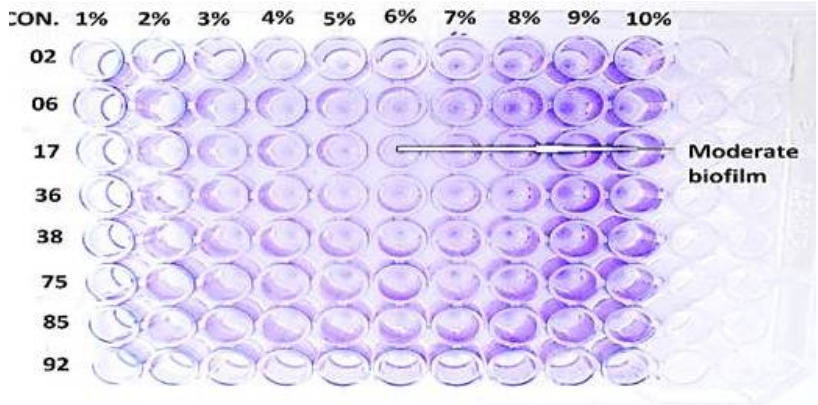
d. Effect of different concentration of Sucrose on biofilm production at 30°C



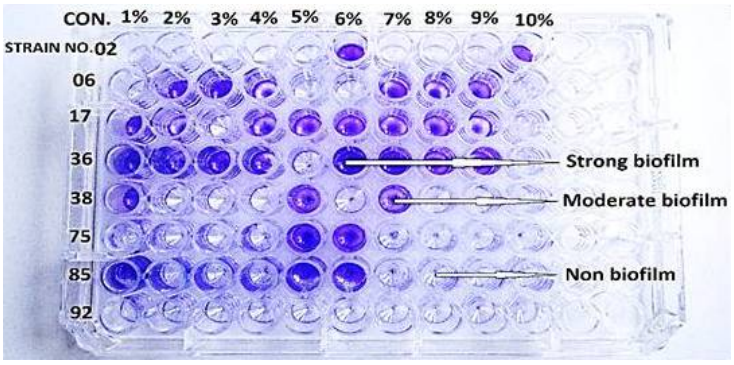
e. Effect of different concentration of Raffinose on biofilm production at 30°C



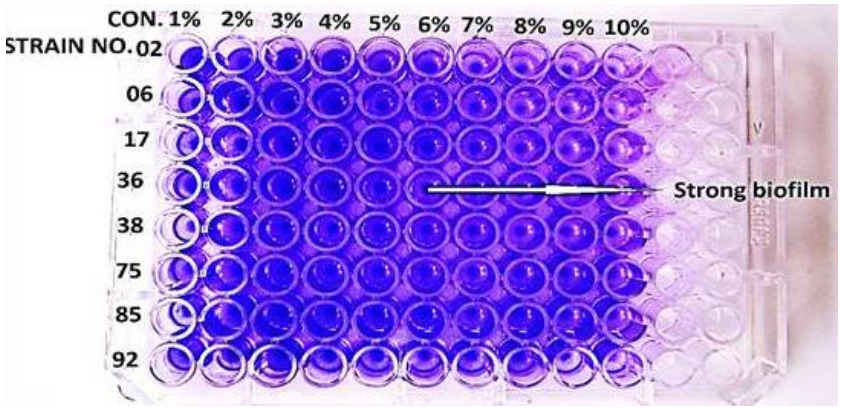
f. Effect of different concentration of Galactose on biofilm production at 37°C



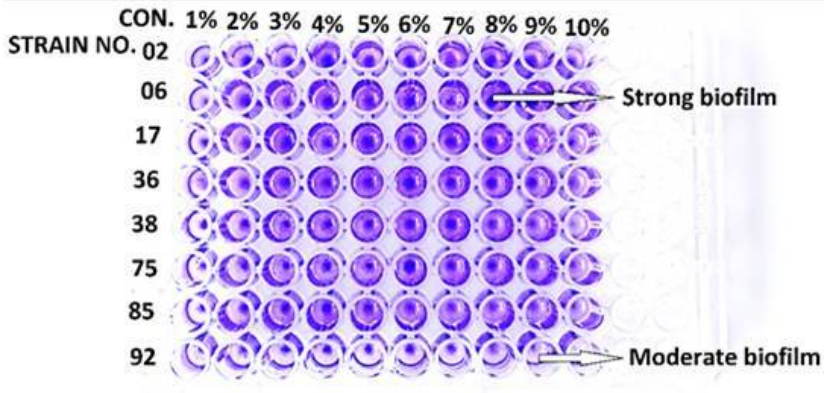
g. Effect of different concentration of Fructose on biofilm production at 37°C



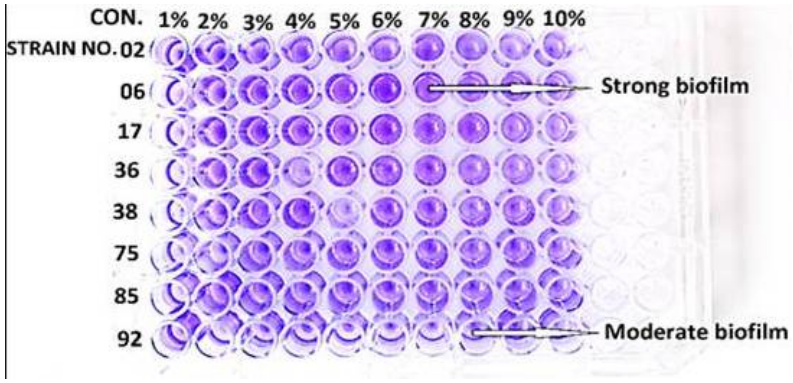
h. Effect of different concentration of Glucose on biofilm production at 37°C



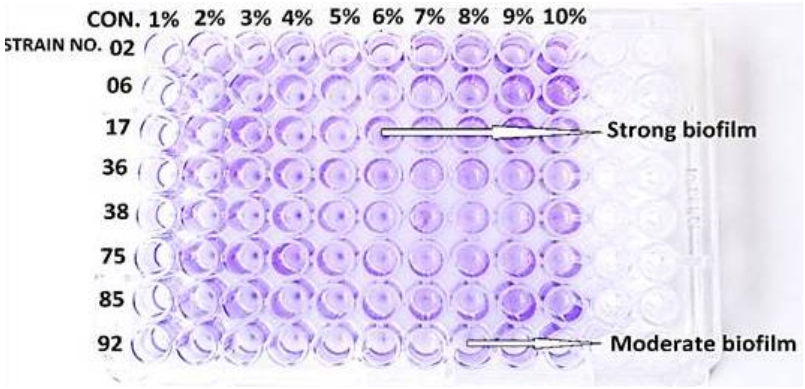
i. Effect of different concentration of Sucrose on biofilm production at 37°C



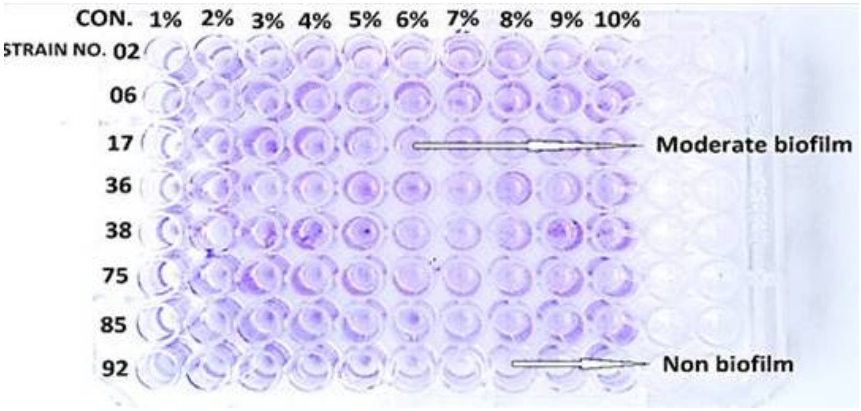
j. Effect of different concentration of Raffinose on biofilm production at 37°C



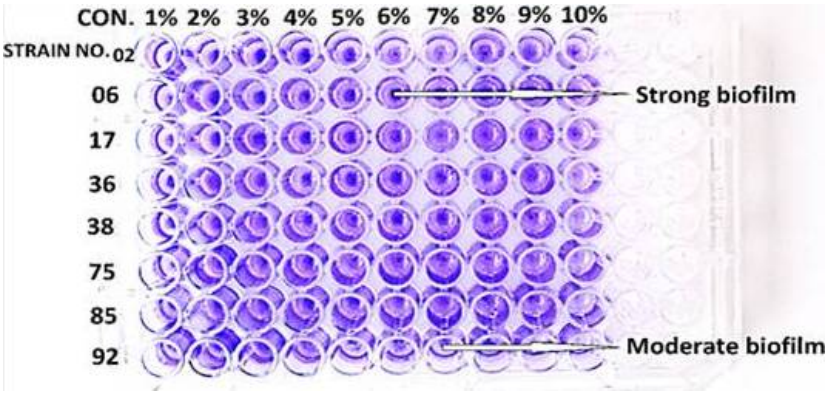
k. Effect of different concentration of Galactose on biofilm production at 45°C



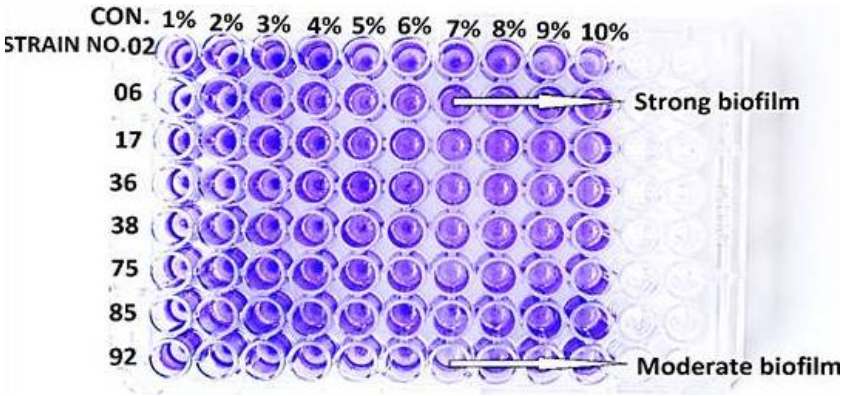
l. Effect of different concentration of Fructose on biofilm production at 45°C



m. Effect of different concentration of Glucose on biofilm production at 45°C



n. Effect of different concentration of Sucrose on biofilm production at 45°C



o. Effect of different concentration of Raffinose on biofilm production at 45°C

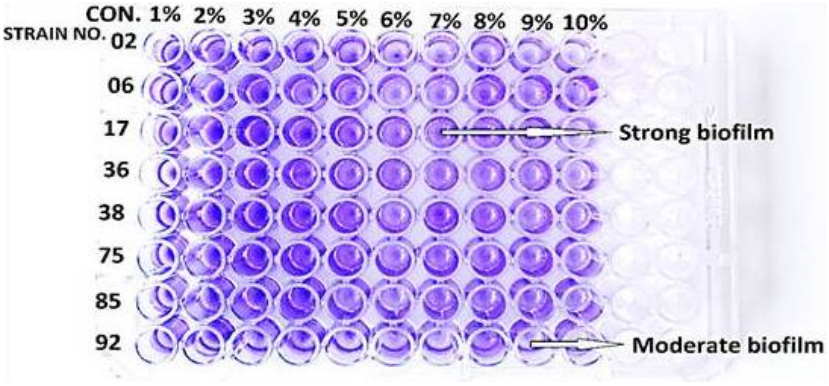
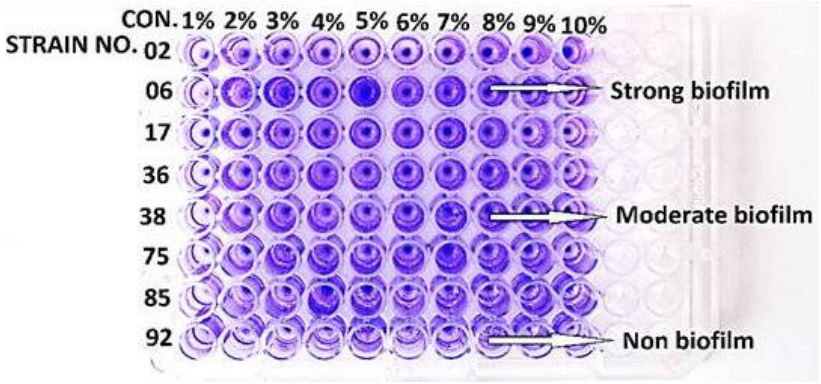
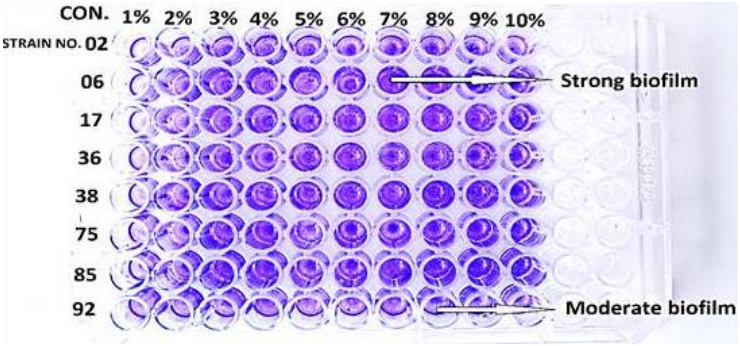


Fig. 8: Effect of nitrogen source on biofilm production by *Lactobacillus acidophilus*

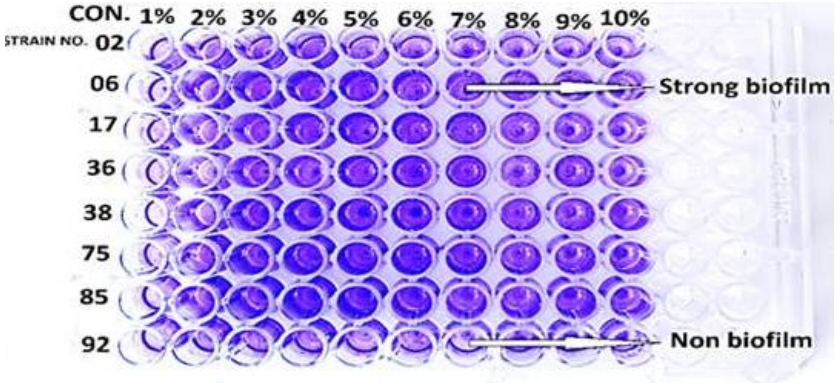
a. Effect of different concentration of Peptone on biofilm production at 30°C



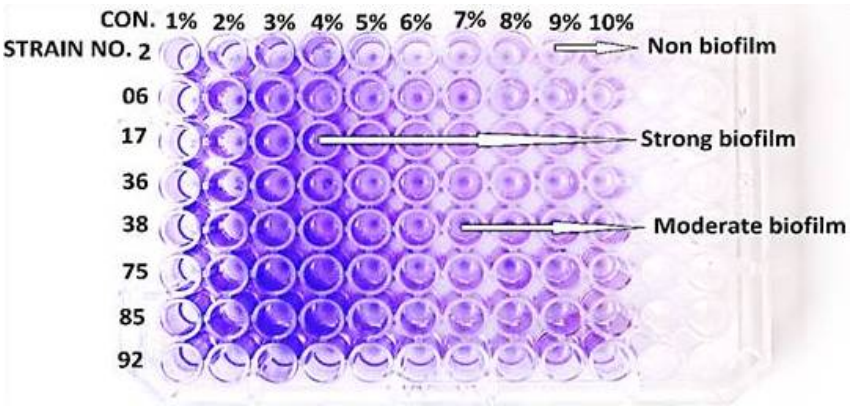
b. Effect of different concentration of Beef on biofilm production at 30°C



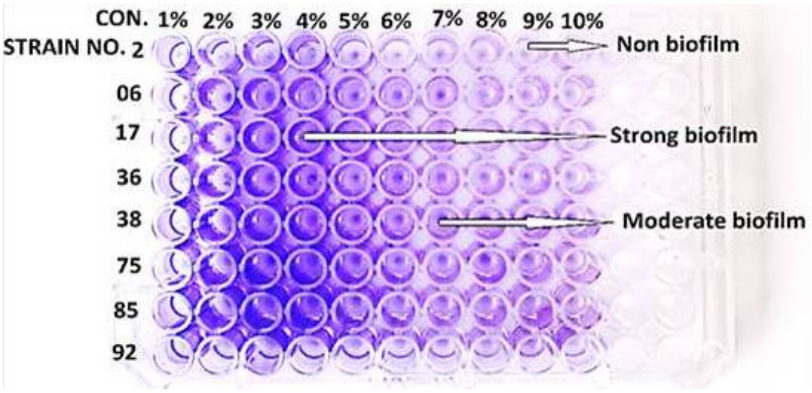
c. Effect of different concentration of Yeast on biofilm production at 30°C



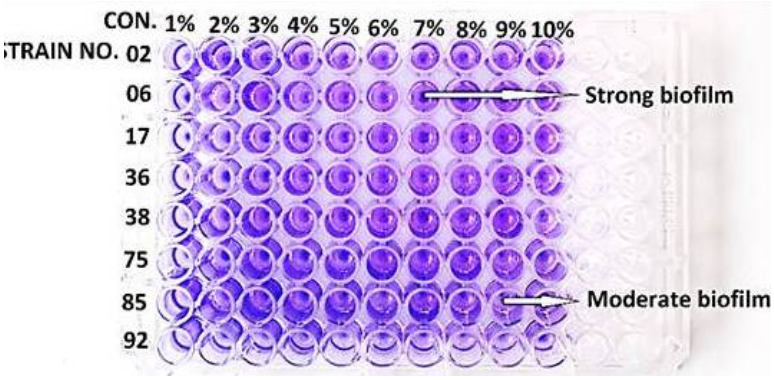
d. Effect of different concentration of Peptone on biofilm production at 37°C



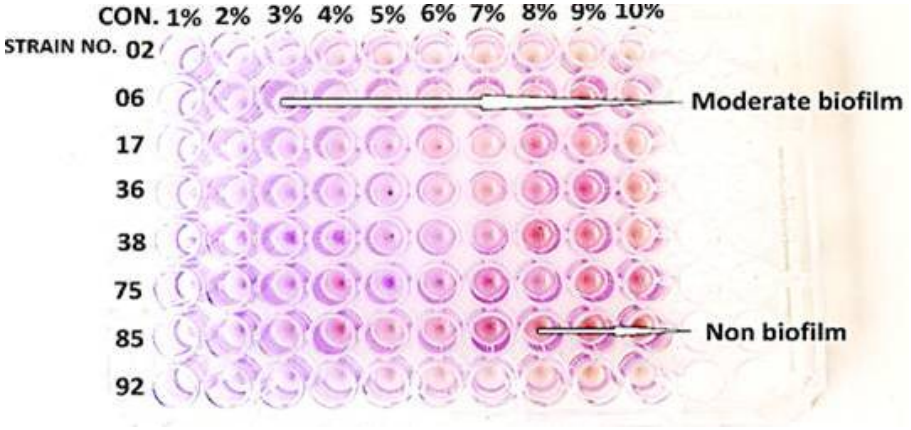
e. Effect of different concentration of Beef on biofilm production at 37°C



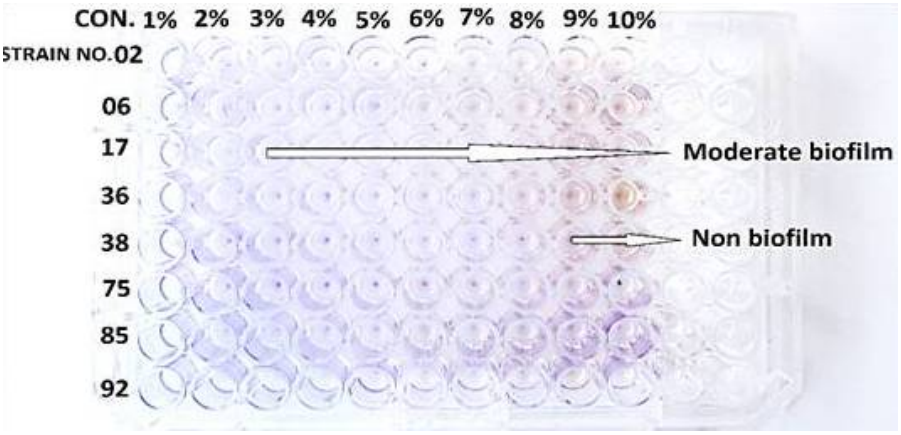
f. Effect of different concentration of Yeast on biofilm production at 37°C



g. Effect of different concentration of Peptone on biofilm production at 45°C



h. Effect of different concentration of Beef on biofilm production at 45°C



Antibiotic susceptibility test

All the 50 cariogenic *Lactobacillus* spp. isolates were tested *invitro* to determine their antibiotic susceptibility patterns by antibiotic disc diffusion method. Totally 10 antibiotic discs were used to identify drug resistant *Lactobacillus acidophilus*. All the isolates showed multiple antibiotic resistances to the antibiotic tested. The maximum resistant pattern percentage (100%) was recorded in strain no LAVG22 and the minimum resistant pattern percentage (10%) was recorded in strain no LAVG01. The antibiotic susceptibility patterns of *L.acidophilus* are shown in Table: 1.

Table 1: Antimicrobial susceptibility pattern of *Lactobacillus acidophilus*

S. No	Antimicrobial agent	Susceptibility pattern	*L. acidophilus*
1.	Rifampicin	S	49
		R	01
2.	Azithromycin	S	41
		R	09
3.	Vancomycin	S	30
		R	20
4.	Amoxicillin	S	46
		R	04
5.	Sulphamethizole	S	29
		R	21
6.	Roxithromycin	S	41
		R	09
7.	Cloxacillin	S	01
		R	49
8.	Ciprofloxacin	S	45
		R	05
9.	Clarithromycin	S	43
		R	07
10.	Trimethoprim	S	16
		R	34

Discussion

Isolation of cariogenic Lactobacillus acidophilus

The plaque samples were collected from tooth decay of rural school children's using sterile forceps around Tirupur District and processed for isolation of exopolysaccharide producing cariogenic *Lactobacillus acidophilus*. The microbial colonies were counted in plaque samples by standard plate count. The analysis of *Lactobacilli* by culture under micro aerophilic conditions in 65 deep caries samples indicated that *Lactobacillus acidophilus* was numerically dominant as compared to other cariogenic isolates. This is due to the presence of *Lactobacilli* in the oral cavity which depends on numerous factors such as the presence of ecological niches e.g. natural anfractuosities of the teeth [5], partly erupted third molars or orthodontic device.

Identification of cariogenic Lactobacillus acidophilus

The isolated bacteria were primarily identified on the basis of the Gram staining, IMViC test, oxidase test, nitrate reduction test, motility test, different carbohydrate fermentation test, starch hydrolysis, growth at different *p*H, and different temperature in the Lactobacilli de Mann Rogosa

and Sharpe (MRS) broth as described as Bergy's Manual of Systematic Bacteriology. Nigatu et al., (2000) [6] stated that since such biochemical methods depend on environmental and culture conditions, they sometimes lead to ambiguous results or even misidentifications. Quere *et al.,* (1997) [7] reported that the increasing number of *Lactobacillus* strains with only slight variations makes the task more difficult.

Optimization of biofilm production by decay causing Lactobacillus acidophilus

The ability of biofilm formation assay in presence and absence of some dietary carbohydrates showed that more biofilm formation is related to presence of the sugars. Numerous studies have been established the role of sugars in caries etiology and the importance of sugars as the principal dietary substrate that drives the caries process [8]. In present study, highest biofilm formation was induced in the presence of glucose (Conc. @ 10% (w/v) / 200µL) by the isolate *L. acidophilus*. This result is in concurrence with the observation laid down by Christensen *et al*., (1985) [9] who found that bacterial adherence and glycocalyx formation was enhanced with the supplementation of glucose in the culture media**.** These carbohydrates may induce the expression of some proteins that are responsible for bacterial adherence. Buhler *et al*., (1998) [10], demonstrated that *E.coli* biofilm formation increased with increasing glucose concentrations and the results indicate that higher glucose concentrations may be beneficial for biofilm formation, independently of the hydrodynamic conditions in microtiter plates. Although in this study, enhanced biofilm formation was observed with increasing concentration of glucose in the medium at 37°C and it was seen to gradually diminish at 45°C. This indicates that biofilm formation was increased to a certain level of glucose concentration in the medium and gradually diminishes at higher temperature. This may be an effect of glucose concentration and higher temperature on the microbe.

In the present study, the response of biofilm formation of the selected isolate with the utilization of different organic nitrogenous compounds used as nitrogen source, it was found that yeast extract was suitable nitrogen source for biofilm formation (Conc. @ 10% (w/v) / 200µL). However, Lawrence et al., (1987) [11], found peptone as the best nitrogen source for biofilm formation by *Pseudomonas fluorescence.* These results suggest that the effect of nitrogenous compound in the formation of biofilm differ from strain to strain.

The optimum temperature and pH for biofilm formation of the isolate were found at 37ºC and 6.5 respectively. The effect of pH values on biofilm formation was found significant. Tang et al., (2012) [12] reported that, in case of *S. aureus* the rise or drop in the *p*H value ($\geq$ 12 or 4 $\leq$) was directly involved in the decrease of biofilm formation. In the present study the results also suggests that extremely low or high *p*H value effect on the biofilm formation.

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